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Title of Invention1 Infectious cDNA clone of GB Virus B and Uses ThereofField of Invention

5 The present invention relates to nucleic acid
sequence which comprises the genome of an infectious GB
virus B (GBV-B) clone. The invention also relates to
the use of the nucleic acid sequence of the infectious
10 GB virus B clone to study indirectly the molecular
properties of hepatitis C virus (HCV), and in the
production of HCV/GBV-B chimeras. The invention further
relates to the use of the infectious nucleic acid
sequence of the GB virus B clone and the HCV/GBV-B
15 chimeras in the development of vaccines and therapeutics
for HCV.

Background of Invention

20 Transmission studies of potential human
hepatitis agents were first reported in 1967 (Deinhardt
1967). Four tamarins inoculated with acute phase sera
from a surgeon with acute hepatitis (patient GB)
developed hepatitis, as did most tamarins inoculated in
serial passage studies. Subsequent studies indicated
25 that the etiological agent responsible for the
development of hepatitis in these animals was not any of
the known human hepatitis viruses (Purcell 1994). In
1995, two related RNA viruses named GB virus-B (GBV-B)
and GB virus A (GBV-A) were identified in acute phase
30 sera of a tamarin which developed hepatitis following
inoculation with serum of the eleventh tamarin passage
of the putative GB agent (Simons 1995a).

35 GBV-B infection of tamarins resulted in acute
resolving hepatitis (Schlauder 1995, Buhk 1997). The

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° natural host of GBV-B is still unknown as the virus has not been detected in uninoculated animals or in humans.

GBV-A, on the other hand, is an indigenous tamarin virus rather than a component of the original GB inoculum (Bukh 1997, Erker 1998). Experimental
5 infection of tamarins with GBV-A did not produce hepatitis (Schlauder 1995). A human agent, GBV-C or hepatitis G virus, most closely related to GBV-A, was later identified (Simons 1995b, Linnen 1996). However,
10 it is still not clear whether this virus actually causes hepatitis (Alter 1998, Bukh 1998a). Thus, of the known GB viruses, GBV-B may be the only true hepatitis virus.

Based on analysis of their genomic sequences, GBV-A, GBV-B and GBV-C were classified as members of the
15 *Flaviviridae* family of viruses, and among the known viruses, GBV-B is the virus most closely related to hepatitis C virus (HCV) (Muerhoff 1995, Robertson 1998).

The GBV-B virus contains a positive-sense, single-stranded RNA genome of 9143 nucleotides (nts)
20 (Simons 1995a, Muerhoff 1995). The viral genome of GBV-B consists of a 5' untranslated region (UTR), a single long open reading frame (ORF) and a 3' UTR. Based on
25 known motifs, structural proteins were predicted to be encoded in the 5' portion of the ORF and nonstructural (NS) proteins in the 3' portion of the ORF (Muerhoff 1995). The hydropathy plots of the polyproteins of GBV-B and HCV are very similar even though the overall
30 homology of the predicted polyproteins between GBV-B and HCV is only about 25-30% (Muerhoff 1995). The putative envelope proteins (E1 and E2) of GBV-B and HCV share common structural features, and significant homology was
35 observed between the NS3 serine protease, the NS3 RNA

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° helicase, and the NS5 RNA-dependent RNA polymerase regions of GBV-B and HCV (Muerhoff 1995). Furthermore, the function and substrate specificity of the GBV-B and HCV NS3 serine proteases are also similar (Scarselli 5 1997). The genomic structure and organization of GBV-B and HCV share additional features of interest. First, colinear regions with significant sequence homology were identified in the 5' UTRs (Muerhoff 1995) and the predicted IRES structure of GBV-B is similar to that of 10 HCV (Lemon 1997). Second, both viruses begin the 3' UTR with a short sequence followed by a poly (U) stretch followed by additional nucleotides (50 nucleotides for GBV-B and 98 nucleotides for HCV). However, the 3' 15 terminal sequence of HCV forms a stable stem-loop structure (Kolykhalov 1996) whereas the published 3' terminal sequence of GBV-B does not.

To date, molecular studies of HCV are severely limited by the lack of an efficient cell culture system 20 for the virus and by expense and limited availability of chimpanzees, the sole animal model for HCV. Accordingly, a less expensive and more readily available animal than chimpanzees is necessary as an animal model 25 for the study of HCV.

Summary of Invention

The present invention relates to nucleic acid sequence which comprises the genome of an infectious GB 30 virus B (GBV-B) clone. It is therefore an object of the invention to provide nucleic acid sequence which encodes an infectious GBV-B. Such nucleic acid sequence is referred to throughout the application as "infectious 35 nucleic acid sequence".

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° As significant structural homology exists between the genomes of GBV-B and HCV, the invention also relates to the use of infection of tamarins with the infectious nucleic acid sequence of GBV-B or with
5 mutants of the infectious sequence to study indirectly the molecular properties of hepatitis C virus (HCV) or as a preliminary screen to identify agents which have antiviral activity against HCV.

10 The invention further relates to "chimeric nucleic acid sequences" consisting of portions of the infectious nucleic acid sequence of GBV-B and portions of the nucleic acid sequences of other viruses closely related to GBV-B such as HCV, GBV-C or other members of
15 the *Flaviviridae* family which do not replicate in tamarins. In a preferred embodiment, the chimeric nucleic acid sequences consist of portions of the infectious nucleic acid sequence of GBV-B and portions of the nucleic acid sequence of HCV. The nucleic acid
20 sequences taken from GBV-B and HCV can be open-reading frame sequences, and/or sequences from the 5'UTR and/or 3'UTR.

In one embodiment, GBV-B/HCV chimeras may be
25 made in which 5' or 3' UTR sequences of the GBV-B infectious clone are replaced with the corresponding sequence from an HCV clone.

In another embodiment, GBV-B/HCV chimeras may be constructed in which the structural or non-structural
30 regions of GBV-B are replaced by corresponding regions of HCV. Thus, such a chimera would contain, for example, the HCV structural region in a GBV-B "genomic backbone". Of course, it is understood by one of skill
35 in the art that the construction of the above-described

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° chimeric nucleic acid sequences may be reversed such that, for example, the GBV structural region may replace the structural region of an HCV genome to produce a chimera in which the GBV structural region is contained in an HCV backbone.

5 The invention further relates to the use of the chimeric nucleic acid sequences of the invention to study the functions of HCV genes, and for the development of vaccine and antiviral agents against HCV.

10 The invention also relates to the use of the infectious GBV-B nucleic acid sequence, the mutated GBV-B nucleic acid sequences or the chimeric sequences of the invention to identify cell lines capable of supporting the replication of GBV-B or the chimeras of the invention.

15 The present invention also relates to the polypeptides encoded by the nucleic acid sequences of the invention or fragments thereof.

20 The present invention further relates to the in vitro and in vivo production of GBV-B, mutant GBV-B viruses or chimeric GBV-B/HCV viruses from the nucleic acid sequences of the invention.

25 The invention also provides that the nucleic acid sequences and viruses of the invention be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

30 Brief Description Of Figures

Figure 1 shows a flow diagram of GB virus transmission studies in two species of tamarins, *Saguinus mystax* (SM) and *Saguinus oedipus* (SO). The animals infected with GBV-B (Simons 1995a) are boxed.

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Two serum pools (GB 8/93 and GB 2/94) were made from acutely infected animals. Both pools contained GBV-B, as well as GBV-A (Simons 1995) at a titer of 10^8 genome equivalent (GE)/ml. A 10% liver homogenate (CT 11/91) was made from a sacrificed tamarin. A number of *S. mystax* tamarins (SM 737, 749, 750, 760, 782, 795 and 799) and *S. oedipus* tamarins (SO 100) were naturally infected with GBV-A_{SM} and GBV-A_{SO}, respectively, prior to inoculation (Bukh 1997). Only two tamarins (SM 720 and 748), both GBV-A_{SM} negative, became infected with GBV-A (Simons 1995) following inoculation. Tamarins SM42 and SM670 were not tested for GBV-A or GBV-A_{SM}.

Figure 2 shows the course of GBV-B infection in tamarins (*S. mystax*) inoculated with a dilution series of the GB 2/94 pool. All animals were inoculated intravenously at week 0 with 1 ml of the indicated dilution. Results of qualitative RT-nested PCR for GBV-B in serum are shown at the top (filled circles, positive; empty circles, negative). Serum levels of isocitrate dehydrogenase (ICD in units/ml; shaded area) and the estimated \log_{10} GBV-B GE titer (vertical columns) were plotted against time.

Figure 3 shows alignment of the 3' UTR sequences of GBV-B. The sequence of the infectious clone of GBV-B (pGBB) is shown at the top (nts. 9038-9399). The other sequences shown are: pGBB5-1, a non-infectious clone of GBV-B; GBV-B, a prototype of GBV-B (Simons 1995); eleven "gb" clones obtained from CT 11/91 liver homogenate by 5' RACE on the minus-strand GBV-B RNA; four "29" clones obtained from GB 2/94 pool by RT-PCR across 5'-to-3'-end-ligated viral GBV-B RNA; and seven "GBB3" clones obtained from GB 2/94 pool by standard RT-PCR.

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With pGBB as the reference, nucleotide substitutions or insertions are shown as uppercase letters, identical nucleotides are shown as dots and nucleotide deletions are shown as dashes.

Figure 4 shows the predicted secondary structure of the 3' UTRs of GBV-B and HCV as determined by the program "mfold" (Genetics Computer Group).

Figure 5 shows the course of GBV-B infection in *S. mystax* tamarins transfected with RNA transcripts of pGBB. Both animals were negative for GBV-A_{SM}. At week 0 transcription mixtures were injected into tamarins by percutaneous intrahepatic injection guided by ultrasound. Results of qualitative RT-nested PCR for GBV-B in serum is shown at the top (filled circles, positive; empty circles, negative). Serum levels of isocitrate dehydrogenase (ICD in units/ml; shaded area) and the estimated log₁₀ GBV-B GE titer (vertical columns) were plotted against time.

Figures 6A-6F show the nucleotide sequence of the infectious hepatitis C virus clone of genotype 1a strain H77C and Figures 6G-6H show the amino acid sequence encoded by the clone.

Figures 7A-7F show the nucleotide sequence of the infectious hepatitis C virus clone of genotype 1b strain HC-J4 and Figures 7G-H show the amino acid sequence encoded by the clone.

Description of The Invention

The present invention relates to nucleic acid sequence which comprises the genome of an infectious GB virus B (GBV-B) clone. The nucleic acid sequence which comprises the genome of an infectious GBV-B virus is

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° shown in SEQ ID NO:1 and is contained in the plasmid construct pGBB deposited with the American Type Culture Collection (ATCC) on May 28, 1999 and having ATCC accession number PTA-152. The present invention relates
5 to the identification of a 260 nucleotide sequence at the 3' end of the infectious GBV-B clone which is shown in Example 3 to be necessary for the development of the infectious clone.

10 Since GBV-B is the virus most closely related to HCV, the present invention also relates to experimental infection of tamarins with the infectious GBV-B clone of the invention or with mutants of the infectious GBV clone to study indirectly the molecular
15 properties of HCV or as a preliminary screen to identify agents which have antiviral activity against HCV. For example, since the predicted internal ribosome entry site (IRES) structure in the 5'UTR of GBV-B is similar to that of HCV (Lemon 1997), the NS3 serine proteases of
20 GBV-B and HCV have been shown to share substrate specificity in vitro (Scarselli 1997), and the 3'UTRs of HCV (Yanagi 1999) and GBV-B (see Examples) have been shown to be critical for viral infectivity, mutagenesis
25 of these regions in the GBV-B infectious clone may be undertaken to examine IRES function, NS3 serine protease activity or the role of the 3'UTR in viral infectivity in vivo. Where such "mutations" are introduced into the GBV-B clone of the invention to create a "mutated" GBV-B
30 sequence, the mutations include, but are not limited to, point mutations, deletions and insertions. Of course, one of ordinary skill in the art would recognize that the size of the insertions would be limited by the
35 ability of the resultant nucleic acid sequence to be

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° properly packaged within the virion. Such mutations could be produced by techniques known to those of skill in the art such as site-directed mutagenesis, fusion PCR, and restriction digestion followed by religation.

5 Alternatively, given the significant structural homology that exists between the genomes of GBV and HCV, the infectious GBV-B clone may be used to screen for inhibitors of IRES function or viral enzyme activity (for example, NS3 helicase, NS3 protease, NS2-
10 NS3 protease or NS5B RNA polymerase activity). Such inhibitors may be useful as antiviral agents to HCV since viral enzyme activity and IRES function are known to be critical for HCV replication.

15 The effect of such inhibitors on the IRES function or viral activity of the GBV-B encoded by the infectious sequence of the invention may be measured by assays known to those of skill in the art to measure directly or indirectly viral replication or viral
20 pathogenicity. Such assays include, but are not limited to, the measurement of virus titer in serum or liver of an infected tamarin by PCR or the measurement of GBV-B viral protein expression in liver cells of an infected
25 tamarin by immunofluorescence or Western blot. Of course, it is understood that a comparison of results obtained for control tamarins (treated only with infectious nucleic acid sequence) with those obtained for treated tamarins (nucleic acid sequence and
30 antiviral agent) would indicate, the degree, if any, of antiviral activity of the candidate antiviral agent. Of course, one of ordinary skill in the art would readily understand that the tamarins can be treated with the
35 candidate antiviral agent either before or after

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° exposure to the infectious nucleic acid sequence of the present invention.

In yet another embodiment, the invention relates to "chimeric nucleic acid sequences" which consist of portions of the infectious nucleic acid sequence of GBV-B and portions of nucleic acid sequences of viruses which are related to GBV-B such as HCV, GBV-C and other members of the Flaviviridae family which do not infect tamarins. In a preferred embodiment, chimeric nucleic acid sequences consist of portions of the infectious nucleic acid sequence of GBV-B and portions of nucleic acid sequences of hepatitis C viruses (HCV) of various genotypes or subtypes; preferably portions of nucleic acid sequence of infectious HCV clones of genotypes 1a (ATCC accession number PTA-157; Figures 6A-6F), 1b (ATCC accession number 209596; Figures 7A-7F) or 2a (ATCC accession number PTA-153; SEQ ID NO: 4). The nucleic acid sequences taken from GBV-B and HCV can be open-reading frame sequences, and/or sequences from the 5'UTR and/or 3'UTR. The gene borders of the HCV genome, including nucleotide and amino acid locations, have been determined, for example, as depicted in Houghton, M. (1996), and the putative gene borders of the GBV-B are shown in Table 1.

Of course, it is understood that the production of GBV-B/HCV chimeras could include insertion of specific genes or regions of the infectious GBV-B clone into an HCV "genomic backbone" (where the HCV genomic backbone is preferably an infectious nucleic acid sequence of HCV genotypes 1a, 1b or 2a described above) or alternatively, could include insertion of

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° specific genes (or portions thereof) or regions of an HCV genome into the GBV-B infectious clone of the invention. Of course, where HCV genes or regions are to be inserted into the GBV-B infectious clone, it is to be understood that the inserted HCV sequences may be
5 unmodified or may be mutated in order to examine the effect of the mutation(s) on the function of the inserted HCV gene or region in the chimeric GBV-B-HCV virus.

10 Such chimeras can readily be produced by methods known to those of ordinary skill in the art.

In one embodiment, GBV-B/HCV chimeras may be made in which 5' or 3' UTR sequences of the GBV-B
15 infectious clone are replaced with the corresponding sequence from an HCV clone. For example, chimeras may be constructed in which the IRES sequence of the infectious GBV-B clone is replaced by the IRES sequence of HCV. Such chimeras can be used in identifying
20 inhibitors of IRES activity which would be useful as antiviral agents, or could be used to examine HCV IRES function in vivo. Alternatively, mutations could be introduced into the HCV IRES contained in the GBV-B
25 clone in order to examine the effect of the mutation(s) on IRES function in vivo.

Alternatively, GBV-B/HCV chimeras may be made in which the 3'UTR sequence of GBV-B is replaced by the
30 3'UTR sequence of HCV. As the 3' terminal stem-loop structure is believed to be important for initiation of RNA replication and has been shown to be critical for infectivity of HCV in vivo, such chimeras may be used for more detailed analysis of the function of the 3' UTR

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° sequence of HCV in vivo and for the testing of candidate antiviral agents.

5 In another embodiment, GBV-B/HCV chimeras may be constructed in which the structural or non-structural regions of GBV-B are replaced by corresponding regions of HCV. Such chimeras would be useful in identifying whether the inability of HCV to infect tamarins is due to the inability of HCV's structural region to bind the receptor necessary for infection of tamarins or to the
10 absence of sequences in HCV's nonstructural regions which are necessary for replication in tamarins. For example, the ability to infect tamarins with GBV-B/HCV chimeras in which the non-structural region of GBV-B is
15 replaced by the non-structural region of HCV would indicate that the structural genes of GBV-B are necessary for viral infection in tamarins, and that the inability of HCV to infect tamarins is likely due to its lack of receptors for HCV.

20 Alternatively, the ability to infect tamarins with GBV-B/HCV chimeras in which the structural region of GBV-B is replaced by the structural region of HCV would indicate that the non-structural genes of GBV-B
25 are critical for viral infection in tamarins, and that the inability of HCV to infect tamarins is likely due to HCV's lack of nonstructural sequences which are necessary for replication in tamarins.

30 Of course, GBV-B-HCV chimeras may be constructed in which only a portion of the non-structural or structural regions of GBV-B are replaced by the corresponding portions of HCV sequences. For example, a chimera in which only one or two of the three
35 structural genes (C, E1 and E2) of GBV-B are replaced by

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° the corresponding HCV structural genes may be made. In one embodiment, nucleic acid sequences comprising the E1 and E2 genes of GBV-B may be replaced by the sequences comprising the HCV E1 and E2 genes. In another
5 embodiment, nucleic acid sequence comprising either the E1 or E2 gene of GBV-B is replaced by sequence encoding either the HCV E1 or E2 gene.

Alternatively, only a fragment of a GBV-B structural gene in the infectious GBV clone may be
10 replaced with the corresponding HCV gene fragments. For example, the amino terminal of the GBV-B E1 gene may be replaced by the corresponding portion of an HCV E1 gene or an amino terminal portion of the GBV-B E2 gene may be
15 replaced by an amino terminal portion of HCV E2 gene tht containing the HVR1 region. As the structural genes of HCV are believed to be important for neutralization, chimeras containing an HCV structural gene(s) or fragment(s) thereof can be used to develop vaccines
20 against HCV.

In yet another embodiment, chimeras in which individual non-structural genes of GBV-B, such as NS3 RNA helicase, NS3 protease, or the NS5B RNA-dependent
25 RNA polymerase are replaced by the corresponding non-structural genes of HCV may be constructed. Such chimeras would, for example, be useful in identifying inhibitors of viral enzyme activity which would be useful as antiviral agents. Of course, it is understood
30 that in order to construct chimeras in which the polyprotein cleavage sites of the GBV-B remain intact, it may be desirable to replace only a fragment of a nonstructural gene of GBV-B with the corresponding HCV
35 gene fragment.

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° The present invention also relates to polypeptides encoded by the nucleic acid sequences of the invention or fragments thereof. In one embodiment, said polypeptide or polypeptides may be fully or
5 partially purified from viruses produced by cells transfected with the nucleic acid sequences of the invention. In another embodiment, the polypeptide or polypeptides may be produced recombinantly from a fragment of the nucleic acid sequences of the invention.
10 In yet another embodiment, the polypeptides may be chemically synthesized.

The present invention further relates to the in vitro and in vivo production of GBV-B, mutated GBV-B
15 or chimeric GBV-B/HCV viruses from the nucleic acid sequences of the invention.

In one embodiment, the sequences of the invention can be inserted into an expression vector that functions in eukaryotic cells. Eukaryotic expression
20 vectors suitable for producing high efficiency gene transfer in vivo are well known to those of ordinary skill in the art and include, but are not limited to, plasmids, vaccinia viruses, retroviruses, adenoviruses
25 and adeno-associated viruses.

In another embodiment, the sequences contained in the recombinant expression vector can be transcribed in vitro by methods known to those of ordinary skill in
30 the art in order to produce RNA transcripts which encode the GBV-B of the invention. The GBV-B of the invention may then be produced by transfecting cells by methods known to those of ordinary skill in the art with either the in vitro transcription mixture containing the RNA
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° transcripts or with the recombinant expression vectors containing the nucleic acid sequences described herein.

5 In assaying the ability of the mutated GBV-B sequences or of the chimeric sequences of the invention to infect tamarins, the virulence phenotype of the virus produced by transfection of tamarins with the sequences of the invention can be monitored by methods known in the art such as measurement of liver enzyme levels (alanine aminotransferase (ALT) or isocitrate
10 dehydrogenase (ICD)) or by histopathology of liver biopsies.

The present invention also relates to the use of the infectious GBV-B sequence, the mutated GBV-B
15 nucleic acid sequences or the chimeric sequences of the invention to identify cell lines capable of supporting the replication of GBV-B or the chimeras of the invention.

20 Transfection of tissue culture cells with the nucleic acid sequences of the invention may be done by methods of transfection known in the art such as electroporation, precipitation with DEAE-Dextran or calcium phosphate, or incorporation into liposomes.

25 In one such embodiment, the method comprises the growing of animal cells in vitro and transfecting the cells with the nucleic acid of the invention, then determining if the cells show indicia of GBV-B or HCV infection. Such indicia include the detection of viral
30 antigens in the cell, for example, by immunofluorescence procedures well known in the art; the detection of viral polypeptides by Western blotting using antibodies specific therefor; and the detection of newly
35 transcribed viral RNA within the cells via methods such

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as RT-PCR. The presence of live, infectious virus particles following such tests may also be shown by injection of cell culture medium or cell lysates into healthy, susceptible animals, with subsequent exhibition of the signs and symptoms of GBV-B infection.

Suitable cells or cell lines for culturing GBV-B or the chimeric GBV-B-HCV include, but are not limited to, lymphocyte and hepatocyte cell lines known in the art.

Alternatively, primary hepatocytes can be cultured, and then infected; or, the hepatocyte cultures could be derived from the livers of infected tamarins. In addition, various immortalization methods known to those of ordinary skill in the art can be used to obtain cell-lines derived from hepatocyte cultures. For example, primary hepatocyte cultures may be fused to a variety of cells to maintain stability.

The invention also provides that the nucleic acid sequences and viruses of the invention be supplied in the form of a kit, alone or in the form of a pharmaceutical composition.

All scientific publication and/or patents cited herein are specifically incorporated by reference. The following examples illustrate various aspects of the invention but are in no way intended to limit the scope thereof.

EXAMPLES

Materials and Methods

Source of GB virus B

Two tamarin pools VR-806, (American Type Culture Collection) and H205, were used for experimental

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- ° transmission of the GB virus agents to tamarins species *Saguinus mystax* and *Saguinus oedipus*.

Amplification, cloning and sequence analysis of GBV-B

5 Viral RNA was extracted from aliquots of the
GB 2/94 serum pool or CT 11/91 liver homogenate with the
TRizol system (GIBCO/BRL). Primers used in cDNA
synthesis and PCR amplification were based on the
genomic sequence of GBV-B published by Simons et al
10 (Simons 1995) shown in SEQ ID NO:3. Long RT-PCR was
performed using Superscript II reverse transcriptase
(GIBCO/BRL) and the Advantage cDNA polymerase mix
(Clontech) as described previously (Tellier 1996). Four
15 subgenomic regions of GBV-B covering the entire
published sequence (Simons 1995) were amplified from
serum and the PCR products were purified and cloned into
pGEM-9Zf(-) (Promega) or pCR2.1 vector (Invitrogen)
using standard procedures.

20 The 5' terminus of GBV-B was amplified from
serum by using the rapid amplification of cDNA ends
(RACE) with dC or dA tailing (GIBCO/BRL) and GBV-B
specific antisense primers. Two different approaches
25 were used to determine the 3' terminal sequence of GBV-
B. In one approach, GBV-B RNA extracted from serum was
circularized with T4 RNA ligase (Promega) and the 5'-to-
3'-end-ligated viral RNA was amplified in RT-PCR using
specific GBV-B primers. In the second approach, the 5'
30 end of the negative strand GBV-B RNA extracted from the
liver homogenate was amplified using the 5' RACE with dC
tailing and GBV-B specific sense primers. The PCR
products were cloned directly into pCR2.1-TOPO by using
35 the TOPO TA Cloning Kit (Invitrogen).

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° The consensus sequence of GBV-B was determined by direct sequencing of PCR products (nucleotides 1-9078 and nucleotides 9130-9359) and by sequence analysis of the clones (nucleotides 1-7135 and nucleotides 7151-
5 9399). Nucleotide positions correspond to those of the infectious clone (pGBB). Analyses of genomic sequences were performed with GeneWorks (Oxford Molecular Group) (Bukh 1995). To determine whether the GenBank data base contained sequences with homology to the GBV-B 3' UTR
10 sequence identified in the present invention, a "Blast" search was performed. The predicted secondary structure of the GBV-B and HCV 3' UTR sequences were determined by the program "mfold" (Genetics Computer Group).

15 Construction of consensus cDNA clones of GBV-B

First, clone pGBB5-1, a consensus clone of GBV-B 2/94 containing the 3' terminus of GBV-B as published by Simons et al was constructed (Simons
20 1995a). The core sequence of the T7 promoter, a 5' guanosine residue and the sequence of GBV-B (9139 nucleotides) were cloned into pGEM-9Zf(-) vector using *NotI*/*SacI* sites. A *BamHI* site was included at the GBV-B 3' terminus. Digested fragments containing the
25 consensus sequence were purified from subclones and ligated using convenient sites. Next, a second consensus clone of GBV-B, clone pGBB, was constructed by inserting the additional 3' terminal sequence, amplified
30 by PCR from one of the clones obtained by the RACE procedure described above, into pGBB5-1 using *XmaI* (at position 9114) and *BamHI* sites. A *XhoI* site was inserted following the GBV-B 3' terminus. DH5-alpha competent cells (GIBCO BRL) were transformed and
35 selected on LB agar plates containing 100 µg/ml

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ampicillin (SIGMA) and amplified in LB liquid cultures at 30°C for 18-20 hrs (Yanagi 1997). Each cDNA clone was re-transformed to select a single clone, and large-scale preparation of plasmid DNA was performed with a QIAGEN plasmid Maxi kit as described previously (Yanagi 1997). Each clone was genetically stable since the digestion pattern was as expected following retransformation and the complete sequence was the expected one.

Intrahepatic transfection of tamarins with transcribed GBV-B RNA

In 100 µl reactions, RNA was transcribed *in vitro* with T7 RNA polymerase (Promega) from 10 µg of linearized template plasmid. The plasmid pGBB5-1 was linearized with *Bam*HI (Promega) and the plasmid pGBB was linearized with *Xho*I (Promega). The integrity of the RNA was checked by electrophoresis through agarose gel stained with ethidium bromide. Each transcription mixture was diluted with 400 µl of ice-cold phosphate-buffered saline without calcium or magnesium (SIGMA) and then immediately frozen on dry ice and stored at -80°C. Within 24 hours of synthesis, two transcription mixtures were injected into each tamarin by percutaneous intrahepatic injection guided by ultrasound (Emerson, 1992; Yanagi 1998, 1999). If the tamarin did not become infected, the same transfection was repeated once. All transfected animals were negative for GBV-A_{SM} as determined by the protocol described previously (Bukh 1997a).

Monitoring of experimental course in tamarins

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° Serum samples were collected weekly from the tamarins and monitored for liver enzyme levels [alanine aminotransferase (ALT), gamma-glutamyltranspeptidase (GGT), and isocitrate dehydrogenase (ICD)] by standard methods and for GBV-B RNA by a specific reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted from 100 µl of serum using the TRIzol reagent. The RNA pellet was resuspended in 10 mM dithiothreitol (DTT) containing 5% (vol/vol) of RNasin (20-40 u/µl) (Promega). The RT-nested PCR was performed with primers from the 5' UTR of GBV-B (external primer pair: 5'-CCT AGC AGG GCG TGG GGG ATT TCC-3' and 5'-AGG TCT GCG TCC TTG GTA GTG ACC-3'; internal primer pair: 5'-GGA TTT CCC CTG CCC GTC TG-3' and 5'-CCC CGG TCT TCC CTA CAG TG-3'). The reverse transcription was performed with avian myeloblastosis virus reverse transcriptase (Promega) and the external anti-sense primer and nested PCR was performed with AmpliTaq DNA polymerase or AmpliTaq Gold DNA polymerase (Perkin Elmer) as described previously (Bukh 1998a). Specificity was confirmed by sequence analysis of selected DNA products. Each set of experiments included a positive control sample (a 10⁻⁶ dilution of GB 8/93, estimated titer 100 genome equivalent (GE)) and appropriate negative control samples. The genome equivalent (GE) titer of GBV-B in positive samples was determined by RT-nested PCR on 10-fold serial dilutions of the extracted RNA (Bukh 1998a). One GE was defined as the number of GBV-B genomes present in the highest dilution positive in RT-nested PCR. The sensitivity of this RT-nested PCR assay for GBV-B was equivalent to that of our RT-nested PCR assay for HCV (Bukh 1998b), for example, conserved NS3

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° primers which had the same sensitivity for GBV-B as the
5' UTR primers could detect HCV at optimal sensitivity
in samples with known HCV genome titer. Testing for
GBV-A and GBV-A variants was performed by RT-nested PCR
5 assays as described previously (Bukh 1997a).

The consensus sequence of the complete ORF was
determined by direct sequencing of overlapping PCR
products obtained by long RT-nested PCR on serum from
one of the tamarins infected with RNA transcripts as
10 previously described (Yanagi 1997).

Example 1

Transmission of GB Agent in Tamarins

To generate virus pools of the GB agent,
15 tamarins were inoculated intravenously with pooled sera
of the eleventh tamarin passage of this agent (Fig. 1).
Acute phase sera from a *S. mystax* tamarin which
developed hepatitis were pooled (GB 8/93) and inoculated
20 into additional *S. mystax* tamarins to generate a second
pool of acute phase serum (GB 2/94). Both serum pools
contained approximately 10^8 GE/ml of GBV-B and GBV-A. A
10% liver homogenate (CT 11/91) was prepared from a *S.*
25 *oedipus* tamarin which developed hepatitis following
inoculation with the twelfth passage of the GB agent.
The titer of GBV-B in the liver homogenate was
approximately 10^7 GE/ml. The GB 2/94 serum and CT 11/91
liver samples were used as GBV-B cloning sources in the
30 present study.

Inoculation of eight *S. mystax* tamarins with
ten-fold serial dilutions of the GB 2/94 pool
demonstrated that its infectivity titer of GBV-B was 10^8
35 tamarin 50% infectious doses (TID₅₀) (Fig. 2). The five

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° GBV-B infected tamarins all developed acute resolving hepatitis characterized by early appearance of viremia (weeks 1 or 2 p.i.), peak viral titers of 10^7 - 10^8 GE/ml and clearance of viremia after 9-16 weeks (Fig. 2). Two
5 of these tamarins (*S. mystax* 769 and 777) were infected only with GBV-B and were negative for GBV-A and GBV-A_{SM}, whereas the other three tamarins were infected with both GBV-B and GBV-A_{SM}. A *S. mystax* tamarin inoculated with the liver homogenate also developed acute resolving
10 hepatitis with peak GBV-B titers of 10^7 GE/ml and clearance of viremia after 11 weeks. Likewise, four *S. mystax* tamarins inoculated with dilutions of the GB 8/93 pool developed acute resolving hepatitis with clearance
15 of the GBV-B virus after 11-26 weeks. Thus, GBV-B infection in *S. mystax* tamarins is characterized by acute hepatitis, early appearance of viremia, high peak viral titers and viral clearance.

Example 2

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Novel 3' Terminal Sequence of GBV-B

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The consensus sequence of the complete 5' UTR of GBV-B (nucleotides 1-445) was deduced from 13 clones containing nucleotides 1-283 and 3 clones containing
25 nucleotides 31-445. In addition, the entire 5' UTR sequence was determined by direct sequencing of the amplicons. The sequences of the various clones were highly conserved and the consensus 5' UTR sequence of
30 GBV-B from this pool was identical to that of the previously published sequence for GBV-B (Simons 1995a). It is noteworthy that 13 of 15 clones analyzed from the rapid amplification of cDNA ends (RACE) procedure
35 contained the published GBV-B 5' terminus (A residue)

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° and that the same 5' terminus was obtained whether the 5' RACE was performed with dC or dA tailing.

5 The consensus sequence of the ORF (nucleotides 446-9037) was determined by direct sequencing of PCR products obtained using long RT-PCR (Yanagi 1997). In addition, 3 clones containing nts. 446-7135 (one of these clones had a deletion of nts. 3036-3636), 2 clones containing nts. 2019-3373, 5 clones containing nts. 7151-8261 and 7 clones containing nts. 7521-9037 were
10 analyzed. The sequences of GBV-B clones in this pool were very homogeneous. Evidence of micro-heterogeneity was found at only 70 (0.8%) nucleotide and 36 (1.3%) amino acid positions, scattered throughout the ORF. The
15 proportion of amino acid positions with heterogeneity ranged from 0.5-3.2% in different putative gene regions (lowest in NS3 and NS5B; highest in E2 and NS2). The GBV-B ORF sequence differed from the published sequence of GBV-B (Simons 1995) at 34 (0.4%) nucleotide and 12
20 (0.4%) deduced amino acid positions, respectively (Table 1).

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Table 1

Nucleotide and amino acid differences among GBV-B (Simons 1995a), the consensus sequence of GBV-B recovered from a virus pool used as the cloning source (GBV-B, 2/94) and the infectious clone of GBV-B (pGBB).

Genomic Region*	Position nt [aa]	Nucleotide			Amino Acid		
		GBV-B	GBV-B 2/94	pGBB	GBV-B 2/94	pGBB	
5' UTR (1-445)							
C (446-913)							
E1 (914-1489)	1030	C	T	T			
E2 (1490-2641)	1498	T	C (t)	C			
	1628 [395]	G	A (g)	A	V	I (V)	I
	2552 [703]	G	A (g)	A	D	N (D)	N
	2562, 2563 [706]	C, A	A, C	A, C	P	H	H
	2566	T	T	T			
	2625 [727]	C	T	T	A	V	V
NS2 (2642-3385)	2647	C	T (c)	T			
	2816 [791]	C	T	T	L	F	F
	2855 [804]	A	G	G	T	A	A
	3235	A	G	G			
NS3 (3386-5125)	3475**	C	C (t)	T			
	3760	C	T (c)	T			
	4114	C	T	T			
	4117	C	A	A			
	4177	T	C	C			
	4615	C	T	T			
NS4A (5126-5290)							
NS4B (5291-6034)	5329	C	T	T			
	5332	T	C	C			
	5350	A	C	C			
	5455	C	T (c)	T			
NS5A (6035-7267)	6413	T	A (t)	A	L	M (L)	M
	[1990]						
	6577	G	T	T			
	6690	T	C (t)	C	I	T (I)	T
	[2082]						
	6965	T	C (t)	C	S	P (S)	P
	[2174]						
	7015	A	G (a)	G			
	7128	G	A	A	G	E	E
	[2228]						
	7138**	A	A	G			
	7142	A	G	G	T	A	A
	[2233]						
NS5B (7268-9037)	7282	T	C (t)	C			
	7849	C	A	A			
	7852	C	T	T			
	8942	G	A (g)	A	V	I (V)	I
	[2981]						
	8971	T	C	C			
	9026	C	T (c)	T			
3' UTR (9038-9399)	9067	T	C	C			
	Poly(U)	27 nts	11-23 nts	23 nts			
	9134	Deletion	C	C			
	9141-9399	ND	259 nts	259 nts			

*Nucleotide positions corresponding to pGBB. Putative gene borders defined as suggested by homology with HCV (Muerhoff 1995). No homology was observed at the NS2-NS3 junction.

**Positions that differ between the cloning source (GBV-B 2/94) and the infectious clone of GBV-B (pGBB). The change introduced into pGBB at position 7138 introduced an artificial *Sall* site. nd: Not determined. Nucleotides and amino acids shown in parenthesis were found as a minor species in the cloning source (GBV-B, 2/94).

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° The sequence for the 3' UTR is shown in Figure
3. Additional 3' UTR sequence was initially identified
by performing RT-PCR across 5'-to-3'-end-ligated viral
RNA extracted from serum. In all 4 clones with GBV-B
5 sequences, the 5' UTR was truncated compared to the
published sequence (simon 1995a). However, whereas one
clone (29c) had the exact 3' terminus previously
published by Simons et al. (Simons 1995a), the three
10 other clones (29a, 29b, 29d) had 150 additional terminal
nucleotides. Compared with the published sequence, all
four clones had a single nucleotide insertion (C
residue) at position 9134. Next, RACE using dC-tailing
only was performed on the 5' end of the negative-strand
15 RNA extracted from the liver homogenate. All 11 clones
analyzed had additional sequences at the 3' terminus.
Compared with the published GBV-B sequence, two clones
(gb6, gb23) had 259 additional nucleotides, 8 clones
(gb9, gb19, gb20, gb21, gb24, gb25, gb30, gb35) had 236
20 additional nucleotides and 1 clone (gb8) had 232
additional nucleotides. Moreover, all of these clones
had the insertion at position 9134. The 3' UTR
sequences among the various clones were highly conserved
25 (Fig. 3). To demonstrate that the terminal 22
nucleotides found only in clones gb6 and gb23 existed in
circulating viruses, RT-nested PCR was performed on 10-
fold serially diluted RNA extracted from the serum pool
GB 2/94 using an RT and external antisense primer
30 deduced from this sequence. GBV-B RNA was detected at a
dilution of 10^{-7} and the sequence of the amplicon was
identical to the sequence recovered from the liver
homogenate. Thus, the 3' UTR of GBV-B consists of a
35 short sequence of 30 nucleotides followed by a 11-24

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° nucleotide-long poly (U) tract (single C residues were observed in GBV-B from the liver homogenate) and a 3' terminal sequence of at least 309 nucleotides. The new GBV-B 3' UTR sequence did not have significant homology to any of the sequences deposited in the GenBank
5 database. A prediction of the secondary structure of the 3' UTR sequence is shown in Figure 4. The most notable feature of the secondary structure is a highly stable stem-loop structure at the very 3' end consisting
10 of 47 nucleotides.

Example 3

The pGBB Clone of GBV-B is Infectious *in vivo*

15 The infectivity of RNA transcripts from the consensus clone pGBB5-1 which encompassed only the published GBV-B sequence (Simons 1995) was first tested. Within the GBV-B sequence there were no deduced amino acid differences and only 2 nucleotide differences (at
20 nucleotide positions 3475 and 7138) between the consensus sequence of the cloning source (GBV-B 2/94) and the sequence of pGBB5-1 clone. In addition, the 3' UTR of pGBB5-1 had a deletion at nucleotide position
25 9134 and was missing the 3' terminal 259 nucleotides (Fig. 3). Prior to transcription, the pGBB5-1 clone was linearized at the *Bam*HI site with digestion at the exact GBV-B 3' terminus. The RNA transcripts from pGBB5-1
30 were injected into the liver of two tamarins (*S. mystax* 797 and 815). GBV-B RNA was not detected in weekly serum samples collected during 17 weeks of follow-up. As the susceptibility of these two tamarins to GBV-B was subsequently demonstrated by experimental infection
35 using a GBV-B virus pool, the consensus clone pGBB5-1

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° which lacks the 3' terminal sequence of GBV-B is thus not infectious *in vivo*.

Next, the infectivity of RNA transcripts from the full-length consensus GBV-B cDNA clone pGBB was tested. The pGBB clone was identical to the pGBB5-1 clone except in the 3' UTR. Thus, in addition to a 5' UTR of 445 nucleotides, an ORF of 8592 nucleotides encoding 2864 amino acids and a 3' UTR of 103 nucleotides, the pGBB clone also contains an additional 259 nucleotides in its 3' UTR. pGBB was linearized at the *Xho*I site which added an additional C residue at the 3' end of the transcribed GBV-B RNA. When RNA transcripts from the pGBB clone were injected into the liver of two tamarins (*S. mystax* 816 and 817), both tamarins became infected with GBV-B with viremia at week 1 p.i. and peak viral titers of 10^8 GE/ml (Fig. 5). The consensus sequence of PCR products of the complete ORF, amplified from serum obtained during week 2 p.i. from one tamarin (*S. mystax* 817), was identical to the sequence of pGBB, including at the two positions which differed from the consensus sequence of the cloning source and from the published sequence of GBV-B (Table 1). By performing RT-PCR as desired above, it was demonstrated that the very 3' terminal GBV-B sequence of pGBB existed in the circulating viruses in this tamarin. Within two weeks of the transfection both tamarins developed hepatitis with dramatically elevated liver enzyme levels (Fig. 5). Thus, the pGBB clone is infectious *in vivo* whereas the clone pGBB5-1 which lacks the last 259 nucleotides was not.

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